Formosan Journal of Surgery (2011) 44, 205–210

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MINI-REVIEW

Clinical significance of hepatic cancer stem cells

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Received 13 July 2011; received in revised form 27 September 2011; accepted 4 October 2011
Available online 8 December 2011

KEYWORDS
cancer stem cell;
hepatic progenitor cell;
hepatocellular carcinoma;
liver stem cell

Summary The human liver consists of three types of liver cells: mature hepatocytes, cholangiocytes, and bipolar adult hepatic stem/progenitor cells (HPC). These three types of cell are commonly regarded as the primary targets of malignant transformation in the liver, if exposed to carcinogens *in vivo* or *in vitro*. Activation and proliferation of hepatic progenitor cells have been reported in precancerous conditions, such as chronic inflammation (hepatitis B/hepatitis C, alcoholic hepatitis and steatohepatitis). An origin of hepatocellular carcinoma (HCC) from hepatic progenitor cells is currently inferred from the fact that many tumors contain a mixture of mature cells and cells phenotypically similar to hepatic progenitor cells. In our series, there were 42 patients (31 males, 11 females, aged 23–80 years old) with HCC, who accepted liver resection, yielding specimens sufficient for pathological studies. Immunohistochemical studies were made with human monoclonal antibodies against OV-6, CD133, CK-19, CD44, AFP for investigating the HPC. HPC grading was higher in HCC patients with hepatitis B or hepatitis C and lower in those with non-B or non-C hepatitis. As regards the survival of HCC patients based on the grading of cancer stem cells (CSC) within the tumor, the group of Grade 0 showed a more favorable survival rate than that of Grade 1–3. The 1-, 3-, and 5-year survival rates of Grade 0 and Grade 1–3 were 92%, 76%, and 69%, and 63%, 50%, and 50%, respectively ($p = 0.073$). These liver CSC would be more resistant to chemotherapeutic agents than tumor cells with limited proliferative potential. In conclusion, we strongly believe that the contributions of HPC warrant research in patients with HCC. Without determining the characteristics of CSC, it is impossible to propose new treatment strategies.

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doi:10.1016/j.fjs.2011.11.002

1. Introduction

Hepatocellular carcinoma (HCC) is commonly found in Taiwan and its prognosis is unsatisfactory with surgical or non-surgical treatment modalities. Hepatic stem cells are indefinite as cells that have the ability to perpetuate themselves through self-renewal and to differentiate into mature cells by multiple signaling pathways. Histologically, the human liver consisted of three types of liver cells: mature hepatocytes, cholangiocytes, and bipolar adult hepatic stem/progenitor cells (HPC). These three types of cell are commonly regarded as the primary targets of malignant transformation in the liver if exposed to carcinogens *in vivo* or *in vitro*. The most important and useful property of stem cells is that of self-renewal.[1,2] HPC are responsible for the regeneration of hepatocytes or cholangiocytes when the liver is injured. Because of this characteristic, striking parallels can be found between stem cells and cancer cells which may regulate self-renewal. However, cancer cells may include cancer stem cells (CSC) with indefinite potential for self-renewal, that initiate tumorigenesis. The mechanisms directing the transformation of stem cells to CSC and HCC remain to be elucidated. The goal of the current studies is to apply such insight to clinical medicine and to develop a better classification system of liver cancer.

The existence of CSC in solid tumors was first reported in 2003, and it was stated that such a small number of cells as $CD44^{+}CD24^{-}$/low lineage cells from human breast cancer tumors were able to initiate new tumors.[3] More recently, highly tumorigenic cells with distinct surface marker phenotypes were indentified in tumors of the GI tract[4–6] and other solid tumors.[3,7,8] Similarly, liver cancer/progenitor cells in HCC have been identified in previous reports.[9–11] In a study by Zhu et al,[11] $CD133^{+}$ cancer cells possessed stem cell properties, including higher proliferative potential, greater colony-forming efficiency, self-renewal and differentiating capacity than $CD133^{-}$ cells. Therefore, a set of surface molecule markers could more accurately define the cancer stem cell subpopulation responsible for the initiation and progression of HCC.[12,13]

2. Defining features of liver progenitor cells

Four possible hepatic stem cells are identified in the canal of Haering (proximal biliary tree): intralobular bile ducts, periductal "null" mononuclear cells, and peribiliary hepatocytes[14] as shown in Fig. 1. Experimental induction of liver stem/progenitor cells in rodents has been extensively studied in liver chemical injury models and carcinogenesis. The application of a variety of experimental protocols in animal models resulted in activation and proliferation of adult liver stem/progenitor cells, often referred to as oval cells. Oval cells are putative liver stem/progenitor cells and were first described by Opie[15] in 1944, and later by Farber[16] in 1956. Activation and proliferation of hepatic progenitor cells have been reported in precancerous conditions, such as chronic inflammation (hepatitis B/hepatitis C, alcoholic hepatitis and steatohepatitis).[17,18] Therefore, HPC could be a source of cancer stem cells in HCC.

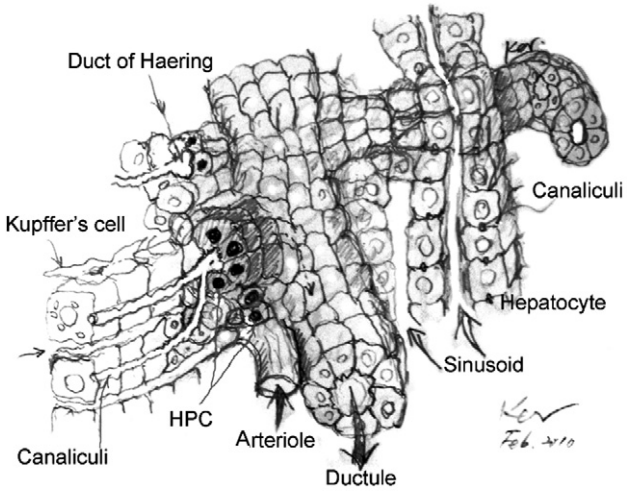

Figure 1 Schema of Duct of Haering and niches of HPC.

3. What are the cancer stem cells?

CSC within a solid tumor give rise to various differentiated tumor cells, and ultimately drive tumor growth and metastasis. The modern tools of stem cell biology have provided strong evidence to show that CSC arise from both normal stem cells and from non self-renewing progenitor cells.[19] Importantly, the term "cancer stem cell" is an operative definition that does not necessarily have a developmental relationship to normal stem cells, but a subset of cells within a tumor can self-renew and elaborate tumor heterogeneity.[20] Ultimately, the definition depends on the assays of self-renewal and tumorigenicity. Some aggressive tumors may have a high percentage of CSC, and chemotherapeutic treatments may increase the frequency of CSC in a tumor because of the non-stem cancer cells killed by chemo agents.[21,22] In addition, sub-clones of CSC will develop into tumorigenic and non-tumorigenic progeny.[23,24] These results suggest that activation of oncogenic pathways in a cellular background of genetic instability, coupled with an inherent ability to self-renew, is involved in the acquisition of metastatic behavior in the CSC population of tumors derived from pluripotent cells.

4. Clinical implication of liver cancer stem cells in hepatocellular carcinoma

HCC could be derived from either the putative periportal stem cells or from the transition duct cells or their progeny (hepatic genitor cells or oval cells). Recent reports have shown that $CD133^{+}$ cells have stem cell characteristics in HCC as well as in other tumors.[25,26] Therefore, we conducted a study to investigate HPC in HCC and their clinical significances. In our department, there were 42 patients (31 males, 11 females, aged 23–80 years old) with HCC, who accepted liver resection and yielded specimens which were sufficient for pathological studies from 2004 to 2007. Immunohistochemical studies were carried out with human monoclonal antibodies against OV-6, CD133, CK-19, CD44

Table 1 Reaction of HPC in HCC with hepatitis B/hepatitis C.

	Hepatitis B ($n = 18$)	Hepatitis C ($n = 15$)	Hepatitis B + C ($n = 1$)	Non-B + C hepatitis ($n = 8$)	Total ($n = 42$)
Mean age	54.50	65.07	65.00	59.50	59.48
Sex M	17 (94.4%)	9 (60.0%)	1 (100%)	4 (50.0%)	31 (73.8%)
F	1 (5.6%)	6 (40.0%)	0	4 (50.0%)	11 (26.2%)
OV-6-n ≥ 1	5 (27.8%)	5 (33.3%)	1 (100%)	1 (12.5%)	12 (28.6%)
OV-6-t ≥ 1	5 (27.8%)	2 (13.3%)	1 (100%)	1 (12.5%)	8 (19.0%)
P-n ≥ 1	9 (50.0%)	11 (73.3%)	0	4 (50.0%)	24 (57.1%)
P-t ≥ 1	6 (33.3%)	4 (28.6%)	0	5 (62.5%)	15 (36.6%)
AFP (mean $\pm$ SD)	20975.04 $\pm$ 83967.29	201.26 $\pm$ 330.90	33.10	1024.90 $\pm$ 2877.73	9257.19 $\pm$ 55049.21
AFP-n ≥ 1	7 (38.9%)	4 (26.7%)	0	1 (12.5%)	12 (28.6%)
AFP-t ≥ 1	6 (33.3%)	4 (26.7%)	0	3 (37.5%)	13 (31.0%)
Survival (M) (mean $\pm$ SD)	41.89 $\pm$ 19.18	32.47 $\pm$ 20.21	34.00	35.63 $\pm$ 21.55	37.14 $\pm$ 19.74

and AFP to investigate HPC in the tumors of HCC patients. The number of HPC cells was counted at 100× magnification under light microscopic examination. The following definitions were used: Grade 0, normal or <25; Grade 1, <25—50; Grade 2, 50—75; Grade 3, >75/field of 100× magnification. Clinical data and its relation with HPC were studied and biostatistical analysis was performed (Table 1). The difference was deemed significant if p value was <0.05. The results are as follows: (1) the architecture of the compartment between hepatocytes and bile ducts including the three cell types, the terminal bile duct cells, transition duct cells and the putative peri-portal liver stem cells, are shown in Figs. 1—3. These cells have been implicated as possibly giving rise to HCC; (2) HGC and hepatitis B and hepatitis C — HPC grading was higher in patients with HCC and hepatitis B or hepatitis C than in those with non-B or C hepatitis, as shown in Fig. 4. The higher incidence of HCC in hepatitis B infection is most probably related to the increased turnover of hepatocytes secondary to destruction of hepatocytes, leading to stimulation of proliferation of stem cells and other progeny early in the hepatocyte de-differentiation proliferation; (3) liver CSC could be identified and showed positive staining with CD133 or OV-6 within the liver cancer tissue, as shown in Fig. 5. The $CD133^{+}$ cells were round in shape and smaller than malignant cells. The 1-, 3-, and 5-year survival rates for Grade 0 and Grades 1—3 were: 92%, 76%, and 69%, and 63%, 50%, and 50%, respectively ($p = 0.073$). The survival rates (Tables 2 and 3) were different due to the various gradings of CSC (Table 4), and the survival rate was better in the group of Grade 0 than that in Grades 1—3, with a non-significant difference as shown in Fig. 6.

5. Discussion

Although the CSC model has existed for over 50 years, only recently have the techniques of modern stem cell biology

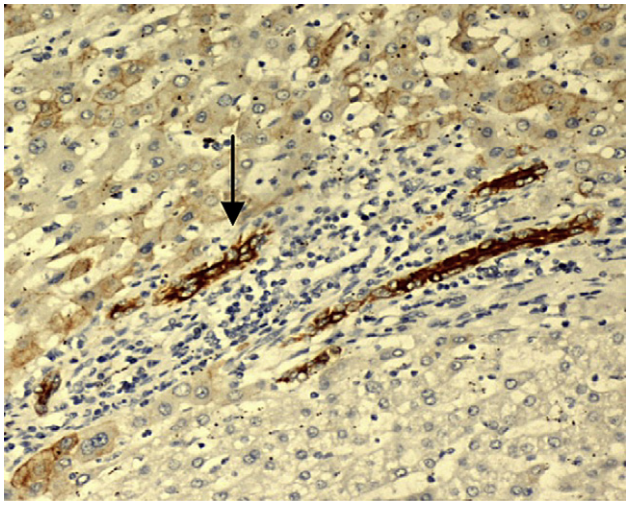

Figure 2 Duct of Haering (arrow) located at non-tumor part and liver progenitor cell (OV-6, 200×).

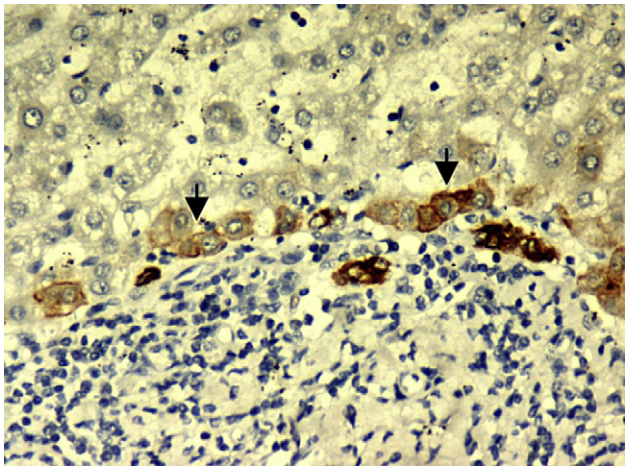

Figure 3 Hepatic progenitor cell(short arrow) and newly formed hepatocytes(OV-6, 400×).

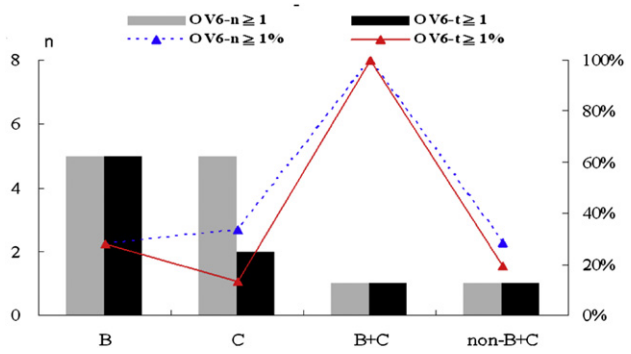

Figure 4 Rate of grading of CSC in the non-tumor and tumor part with hepatitis B/hepatitis C.

Table 2 Survival rate according to the severity of HPC in the non-tumor part of HCC.

	n	1-y death	1-y (%)	3-y death	3-y (%)	5-y death	5-y (%)
(A) OV-6, non-tumor	30	3	90.00	7	75.79	7	75.79
Grade 0							
Grade 1–3	12	3	75.00	5	58.33	6	43.75
(B) OV-6, tumor							
Group 0	34	3	91.18	8	75.70	9	69.39
Group 1–3	8	3	62.50	4	50.00	4	50.00

Log Rank Test: (A) $p = 0.104$; (B) $p = 0.073$.

begun to facilitate rapid progress in this field. It had been demonstrated formally that a single cell can generate a heterogenous cancer and self-renew by the isolated pure tumorigenic cancer stem cells. As pointed out earlier,[17,19] and also in our present results, etiological factors, such as inflammation and viral hepatitis B and C, appear to contribute to the development of HCC by creating phenotypically altered hepatocytes or hepatic progenitor cells. A more recently proposed hepatic progenitor cell model for HCC tumorigenesis may provide a personalized approach for mentioning diagnosis and treatment strategies in these patients. HCC could be derived from progenitor cells or de-differentiated transformed cells, based on the observation that embryonic stem cells and CSC have similar biological behavior. Depending on the extent of genetic alteration, the tumor cells may remain benign or become malignant and even show metastatic potential. This could explain the heterogenicity in HCC morphology, clinical behavior and molecular profiles for tumorigenesis,[27] as shown in Fig. 7. Therefore, the HCC tumor initiated by a multistep carcinogenesis event and by resulting in heterogenity in morphology with stem cells of positive specific phenotypes, appears to have the capability to be more aggressive.[28]

Concerning clinical implications of CSC, HCC expresses some de-differentiation markers in the fetal liver, such as AFP, CD133 or OV-6 (expression) in poorer prognoses.[29,30] In fact, a small population of $CD133^+$ cells could be present in hepatoblastoma as well as in HCC, suggesting the possibility of stem cell origin in both.[31] In a report by Yeh et al,[32] the survival analysis indicated that both CD133 and p53 expression in HCC predict poor disease-free survival ($p = 0.009$ and 0.001), whereas the presence of only CD133 expression predicted poor overall survival ($p = 0.001$). The Cox proportional hazard model showed that p53 and CD133 expression were two independent predictors for disease-free survival. However, the severity of the inflammatory infiltrate in chronic viral hepatitis is correlated with the activation and localization of hepatic progenitor cells.[32,33] Therefore, the confliction of the results of CD133 was correlated with the presence or absence of hepatitis in previous reports.[32,34,35] However, the severity of $CD133^+$ was more strongly expressed in the presence of both hepatitis B and hepatitis C in our studies. The survival rate was worsened by the presence of CD133 in the tumor or non-tumor part in HCC patients, such as OV-6 and CK19 (unpublished data).

In Taiwan, the majority of HCC patients are causatively associated with hepatitis B/C virus infection with cirrhosis. Liver stem cells may play an important role in liver regeneration, and are indented to have accumulating mutations on these progenitor cells, resulting in malignant transformation. The role of progenitor cells in hepatocarcinogenesis is exemplified by chronic hepatitis, advanced liver diseases, and non-alcoholic fatty liver diseases.[34] In our study, about 29% (12/42) and 19% (8/42) of our HCC patients, in non-tumor and tumor part, respectively, have positive expression of one or more markers of progenitor cells such as alfa-fetoprotein, OV-6, CD133 and CK-19. This expression profile can be the result of the acquisition of

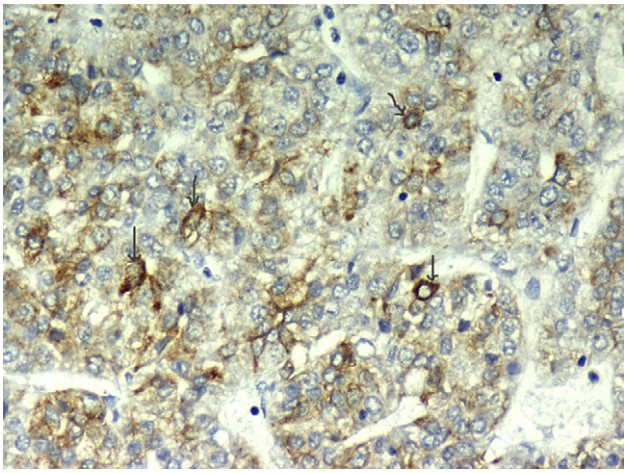

Figure 5 Liver cancer stem cells (arrows) scattered within the tumor of hepatocellular carcinoma (CD133 stain, 400×).

Table 3 Survival rate according to the severity of HPC in the tumor part of HCC.

OV6-tumor	*n*	1-y death	1-y (%)	3-y death	3-y (%)	5-y death	5-y (%)
Group 0	34	3	91.18	8	75.70	9	69.39
Group 1–3	8	3	62.50	4	50.00	4	50.00

Log Rank Test: $p = 0.073$.

Table 4 Grading according to the gender of HCC.

Sex	OV6-n				OV6-t			
	0	1	2	3	0	1	2	3
Male	19 (63.33%)	10 (100%)	1 (100%)	1 (100%)	24(%)	5 (83.33%)	1 (100%)	1 (100%)
Female	11 (36.67%)	0	0	0	10(%)	1 (16.67%)	0	0
Total	30 (71.43%)	10 (23.81%)	1 (2.38%)	1 (2.38%)	34 (80.95%)	6 (14.29%)	1 (2.38%)	1 (2.38%)

progenitor cell markers during malignant transformation, through either the manner of the de-differentiation hypothesis in matured hepatocytes, or the maturation-arrest hypothesis in progenitor cells. If the maturation-arrest hypothesis is correct, and the malignant cells result from gene mutations during maturation from progenitor cells, these HCC precursors should express progenitor cell features histopathologically, as shown in our series.

If the growth of HCC was driven by liver CSC, it would have profound implications on cancer therapy. All phenotypically diverse cancer cells are treated as though they have unlimited proliferative potential and would acquire the ability to metastasize. However, a small number of disseminated cancer cells can be detected at sites distant from primary tumors in patients that never manifest a metastatic status.[36,37] These disseminated, circulating cancer cells will possibly either be killed by the host immune surveillance system, or lack the ability to form a new metastatic tumor. If HCC stem cells can be identified prospectively and isolated, we should be able to identify, more efficiently, new diagnostic markers and therapeutic targets expressed by the liver stem cells. Therefore, we can explain the failure to develop chemotherapies that can consistently eradicate HCC. Although currently available drugs, including chemotherapeutic agents, or agents in target therapies, can shrink metastatic HCC or other metastatic lesions, these effects are usually transient and often do not appreciably prolong the life of patients.[25] One reason for the failure of these treatments is the acquisition of drug resistance by the cancer cells. Another possibility is that existing therapies fail to effectively kill cancer stem cells. Normal stem cells, from various tissues, tend to be more resistant to chemotherapeutic agents than mature cell types from the same tissues.[38] This may be due to high levels of expression of anti-apoptotic proteins or ABC transporters, such as the multidrug resistance gene.[36,38] These liver CSC would be more resistant to chemotherapy than tumor cells with limited proliferative potential. In our un-published data, positive-staining ABC transporters were rich in recognized liver CSC, as shown by tissue immunohistological staining. Liver CSC are more specifically directed against chemotherapy or radiotherapy and the tumor might pare enough cancer stem cells to allow regrowth of the tumor, or so-called recurrence, as shown in Fig. 7. The overall long-term survival of patients with HCC is poor, because in most, HCC is concomitant with chronic liver diseases. Therefore, for HCC patients at all stages, there is a need for prognostic and predictive biomarkers that can aid in the sub-classification of these tumors and help to identify patients in each group who are most likely to respond appropriately to available treatment modality.

In conclusion, self-renewal is the hallmark property of stem cells in normal hepatocytes and HCC. Hepatocytes or cholangiocytes contain self-renewing stem cells, and steps in progression to HCC probably also occur in stem cells.

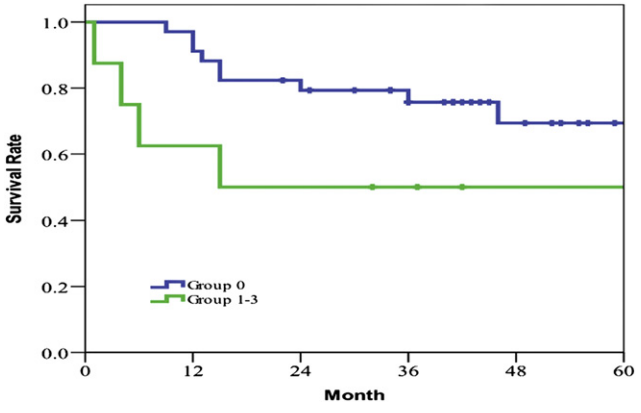

Figure 6 Survival rate of HCC patients based on the grading of CSC within the tumor part and more favorable survival rate in the group of Grade 0 than in Grades 1—3 (Log Rank test, $p = 0.073$).

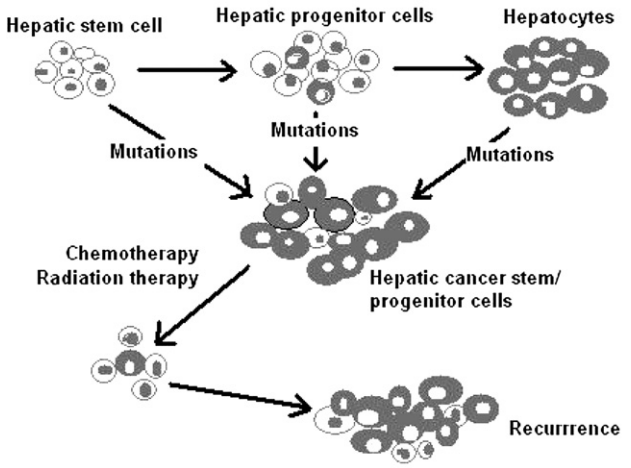

Figure 7 HCC tumorigenesis based on the cancer stem cells model. Liver cancer stem cells can arise from the hepatic stem cells, hepatic progenitor cells or hepatocytes, as a consequence of exposure to various risk factors such as HBV, HCV, aflatoxin and alcohol. In addition, cancer stem cells are resistant to the chemo/radiation therapy and might contribute to recurrence caused by the hepatic cancer stem cells, still alive after treatment.

Liver cancer tumors may be mixed with HPC within cancer tissue that can renew indefinitely and become different from most stem cells that have limited proliferative potential. In order to cure cancer, it is necessary to kill a sufficient amount of CSC. The survival rates and recurrence rates in HCC, related with HPC, are worth investigating. The contributions of HPC warrant research in patients with HCC. Without investigation of these characteristics of CSC, it would be difficult, if not impossible, to propose new treatment strategies for our patients.

Acknowledgments

The authors would like to express their sincere appreciation to the Cancer Center of Kaohsiung Medical University Hospital and the research fund supported from the Ministry of Health 2010, Taiwan.

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